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Conformational Change of the Coronavirus Peplomer Glycoprotein at pH 8.0 and 37°C Correlates with Virus Aggregation and Virus-Induced Cell Fusion

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We have obtained biochemical and electron microscopic evidence of conformational changes at pH 8.0 and 37°C in the coronavirus spike glycoprotein E2 (S). The importance of these changes is reflected in the loss of virus infectivity, the aggregation of virions, and increased virus-induced cell fusion at the same pH. Coronavirus (MHV-A59) infectivity is exquisitely sensitive to pH. The virus was quite stable at pH 6.0 and 37°C (half-life, ~24 h) but was rapidly and irreversibly inactivated by brief treatment at pH 8.0 and 37°C (half-life, ~30 min). Virions treated at pH 8.0 and 37°C formed clumps and large aggregates. With virions treated at pH 8.0 and 37°C, the amino-terminal peptide $E2_N$ (or S1) was released from virions and the remaining peptide, $E2_C$ (S2), was aggregated. Viral spikes isolated from detergent-treated virions also aggregated at pH 8.0 and 37°C. Loss of virus infectivity and E2 (S) aggregation at pH 8.0 and 37°C were markedly enhanced in the presence of dithiothreitol. On the basis of the effects of dithiothreitol on the reactions of the peplomer, we propose that release of $E2_N$ (S1) and aggregation of $E2_C$ (S2) may be triggered by rearrangement of intramolecular disulfide bonds. The aggregation of virions and the isolated E2 (S) glycoprotein at pH 8.0 and 37°C or following treatment with guanidine and urea at pH 6.0 and 37°C indicate that an irreversible conformational change has been induced in the peplomer glycoprotein by these conditions. It is interesting that coronavirus-induced cell fusion also occurred under mildly alkaline conditions and at 37°C. Some enveloped viruses, including influenza viruses and alphaviruses, show conformational changes of spike glycoproteins at a low pH, which correlates with fusion and penetration of those viruses in acidified endocytic vesicles. For coronavirus MHV-A59, comparable conformational change of the spike glycoprotein E2 (S) and cell fusion occurred at a mildly alkaline condition, suggesting that coronavirus infection-penetration, like that of paramyxoviruses and lentiviruses, may occur at the plasma membrane, rather than within endocytic vesicles.

Fusogenic glycoproteins of several different groups of enveloped viruses undergo pH-induced conformational changes. Alphaviruses and orthomyxoviruses infect host cells by receptor-mediated endocytosis and penetrate the cytoplasm by fusion in acidic endosomes. This fusion appears to be mediated by an irreversible conformational change that requires a pH in the range of 5.0 to 6.0 (15, 19, 33, 44). Envelope glycoproteins of bunyaviruses, rhabdoviruses, and murine retroviruses also exhibit conformational changes at pH 5.0 to 6.0 that render them fusogenic (14, 31, 43).

Paramyxoviruses and human immunodeficiency virus, in contrast, possess fusogenic glycoproteins that are activated at pH 7.0 to 8.0 (25, 29). These viruses may possess the ability to penetrate cells at the plasma membrane directly, without requiring endocytosis.

Coronaviruses such as mouse hepatitis virus (MHV) cause cell fusion in intestinal epithelial cells during infection *in vivo* (2) in the alkaline environment of the small intestine. Therefore, it seemed likely that the pH optimum for coronavirus-induced fusion would be neutrality or alkalinity. It was of considerable interest to study the effects of pH on coronaviruses and to determine whether a pH-dependent conformational change in the peplomeric glycoprotein might play a role in virus-induced cell fusion and/or penetration.

The peplomer glycoprotein of MHV-A59 is responsible for binding to receptors on murine cells (16; S. R. Compton and K. V. Holmes, unpublished data), induction of neutralizing antibodies (7) and cell-mediated cytotoxicity (17, 42), and virus-induced cell fusion (11, 39). The induction of cell fusion by MHV-A59 requires cleavage of the 180,000-molecular weight E2 glycoprotein (180K E2 [or S] glycoprotein) into two polypeptides of approximately equal size, called $E2_N$ (the N-terminal half of E2 [S], alternatively designated 90B or S1) and $E2_C$ (the C-terminal half of E2 [S], or 90A or S2) (4, 11, 39). This cleavage occurs during viral maturation, or it can be produced by treatment of virions with trypsin (36). The two 90K subunits of MHV-A59 have been separated by sodium dodecyl sulfate (SDS)-hydroxyapatite chromatography (32).

In this report, we show that the E2 (S) glycoprotein is exquisitely sensitive to high pH. Release of $E2_N$ (S1) from virions at pH 8.0 and 37°C and aggregation of $E2_C$ (S2) are associated with a loss of virus infectivity and the aggregation of virions. These changes are facilitated in the presence of dithiothreitol (DTT). The observed high pH optimum for coronavirus-induced syncytium formation is consistent with conformational changes in E2 (S) at a high pH playing an important role in virus penetration and cytopathogenicity.

MATERIALS AND METHODS

Cells and virus. The 17 Cl 1 and L2 lines of mouse fibroblasts were propagated as described previously (38). MHV-A59 was propagated in 17 Cl 1 cells and radiolabeled

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between 1 and 28 h postinfection in media containing [^{3}H]fucose (2.5 μCi/ml) or [^{35}S]methionine (3.0 μCi/ml) (New England Nuclear Corp.). Released virus was purified as previously described (38). Equilibrium sedimentation of virus was performed in a 20 to 50% (wt/wt) sucrose gradient in TMEN 6.5 buffer (0.05 M Tris-maleate, 0.01 M EDTA, 0.1 M sodium chloride, pH 6.5) at 24,000 rpm for 18 h in a SW28 rotor (Beckman Instruments, Inc.).

Velocity sedimentation of virions was performed in a 15 to 30% (wt/wt) sucrose gradient with a cushion of 50% (wt/wt) sucrose in TMEN 6.5 buffer at 24,000 rpm for 30 min at 4°C in a SW41 rotor (Beckman).

SDS-polyacrylamide gel electrophoresis (PAGE). Viral proteins were analyzed on cylindrical 10% polyacrylamide gels by using the Laemmli buffer system and fractionated with a Gilson gel fractionator (34). The spacer gel was counted as the first fraction. Material retained at the top of the resolving gel was found in fractions 2 and 3.

Trypsin treatment of virions. Purified virions harvested from a linear 20 to 50% (wt/wt) sucrose gradient were treated with a 10-μg/ml concentration of trypsin-TPCK (treated with *N*-tosyl-L-phenylalanine chloromethyl ketone; Worthington Diagnostics) for 30 min at 37°C in TMEN 6.5 buffer, followed by incubation for 30 min at 4°C with a 50-μg/ml concentration of soybean trypsin inhibitor (Worthington). The virus in sucrose was then diluted with TMEN 6.5 buffer and concentrated by sedimentation through 2 ml of 20% sucrose in TMEN 6.5 in an SW28 or SW41 rotor at 24,000 rpm for 2.5 h at 4°C, and the pellet was suspended in TMEN 6.5 or TMEN 8.0 (pH 8.0) buffer.

pH treatment of virions and isolated NP-40-solubilized E2 (S). Trypsin-treated virions or Nonidet P-40 (NP-40)-solubilized E2 (S), isolated as previously described (38) in sucrose density gradients in TMEN 8.0 (or 6.5) buffer, was diluted in TMEN buffer (pH 8.0 or 6.0) to a final sucrose concentration of 0.4% and then incubated at 37°C for 2 to 20 h. Following pH treatment, virions were concentrated by centrifugation at 45,000 rpm in an SW60 rotor for 60 min at 4°C. The supernatant was saved for analysis of released E2 (S). Isolated or released E2 (S) was concentrated with a Speed Vac dryer (Savant Instruments, Inc.).

Guanidine-urea treatment of isolated E2 (S). E2 (S) solubilized with 0.25 to 0.50% NP-40 from purified, trypsin-treated virions was incubated at pH 6.0 (TMEN 6) or pH 8.0 (TMEN 8) and then dialyzed against greater than 100 volumes of 7 M guanidine–0.05% NP-40–5% β-mercaptoethanol, followed by 9 M urea–0.05% NP-40–5% β-mercaptoethanol for 48 h each at room temperature. For electrophoresis, SDS was added to a concentration of 2%, bromophenol blue was added to a concentration of 0.01%, and samples were incubated at 100°C for 1.5 min, cooled, and reduced with 5% β-mercaptoethanol.

Isolation of peplomers and separation of $E2_N$ (S1) and $E2_C$ (S2). The two 90K subunits of E2 (S) were isolated from trypsin-treated virions by SDS-hydroxyapatite chromatography as previously described (32). Virions were solubilized with SDS (2%) at 37°C or with NP-40 (0.5%) or Triton X-114 (10%) at 4°C. The $E2_N$ (S1) and $E2_C$ (S2) subunits were separated by chromatography on Ultrogel HA (LKB Instruments, Inc., or IBF Biotechnics) or by high-pressure liquid chromatography with Bio-Gel HPHT (Bio-Rad Laboratories) in 0.1% SDS and elution by a gradient of 0.15 to 0.50 M sodium phosphate, pH 6.8.

Cell fusion assay. Confluent monolayers of 17 Cl 1 cells in 20-cm^2 tissue culture dishes were infected with MHV-A59 at a multiplicity of infection of 1 to 10 PFU per cell. The virus inoculum was prepared in 0.5 ml of Eagle minimum essential medium with 10% fetal bovine serum. After adsorption for 60 min at 37°C, the inoculum was removed and 5 ml of buffered medium was added to duplicate cultures at each pH.

Incubation was continued at 37°C without additional CO_2. Minimum essential medium was buffered at pHs 6.5, 6.8, 7.1, 7.4, 7.7, 8.0, and 8.3 as described by Eagle (10). Mock-infected control cultures were prepared at pHs 6.5, 7.1, 7.7, and 8.3. At 8 h postinfection, the pH of the medium was measured and the cell monolayer was fixed by addition of glutaraldehyde to a final concentration of 2%. The cells were photographed under phase-contrast illumination. At least 1,000 nuclei and the associated cells were counted in cultures maintained at each pH. The fusion index (*f*) was defined as $1 - C/N$, where C equals the number of cells and N equals the number of nuclei (43).

RESULTS

Effects of pH 8.0 treatment on coronavirus structure and infectivity. Empirically, it is well known that the infectivity of coronaviruses is most stable at pH 6.0 to 6.5 (1, 30, 35). The molecular mechanism of this observation had not been explored, however. The effect on MHV-A59 infectivity of incubation at 37°C in different pHs over a range from 3.0 to 10.0 is shown in Fig. 1. The virus was most stable at pH 6.0, with a surviving fraction of approximately 0.5 at 24 h. In marked contrast is the effect of treatment at pH 8.0 and 37°C, in which half the infectivity was lost in about one-half hour. This rapid loss of infectivity at pH 8.0 was irreversible, as shown by the fact that the virus assays were all done at pH ca. 7. Extremes of pH, such as pH 3.0 and pH 9.0 or 10.0, inactivated the virus even more rapidly. In this article, we concentrate on the effects of pHs in the physiological range from 6.0 to 8.0.

The striking inactivation of MHV at pH 8.0 was temperature dependent. When virions which had been purified at pH 6.0 and 4°C were incubated at pH 8.0 and 4°C for 24 h, viral infectivity was reduced by only a factor of 4 (35). Thus, the critical event in the irreversible inactivation of MHV-A59 at pH 8.0 requires an elevated temperature (37°C).

To determine how treatment at pH 8.0 and 37°C inactivated the virus, virions purified at pH 6.0 and 4°C and then incubated at either pH 6.0 or pH 8.0 for 120 min at 37°C were analyzed by sedimentation to equilibrium on sucrose density gradients at pH 6.0. Virions incubated at pH 8.0 and 37°C for 120 min had a density of 1.19 g/ml, exactly the same as that of virions held at pH 6.0 (Fig. 2). Virions sedimented on gradients at pH 8.0 gave similar results (data not shown). These data show that although infectivity was lost, virions were not disrupted by treatment at pH 8.0.

However, exposure to pH 8.0 and 37°C produced significant changes in virion structure. When virions in high concentrations (10^9 PFU/ml; 500 μg of virus protein per ml) were held at pH 8.0 and 37°C for 0.5, 2, or 24 h and then compared with virions treated similarly at pH 6.0 by sedimentation velocity in a sucrose gradient, it became apparent that virions treated at 37°C for even 30 min at pH 8.0 were aggregated into large clumps which sedimented onto the cushion at the bottom of the gradient (Fig. 3). In contrast, virions treated at pH 6.0 and 4°C (Fig. 3) or 37°C (data not shown) for 0.5 to 24 h sedimented much more slowly. This observation of virus clumping at pH 8.0 and 37°C was confirmed by electron microscopic observation of virions gradient purified at pH 6.0 and 4°C and then incubated for 30

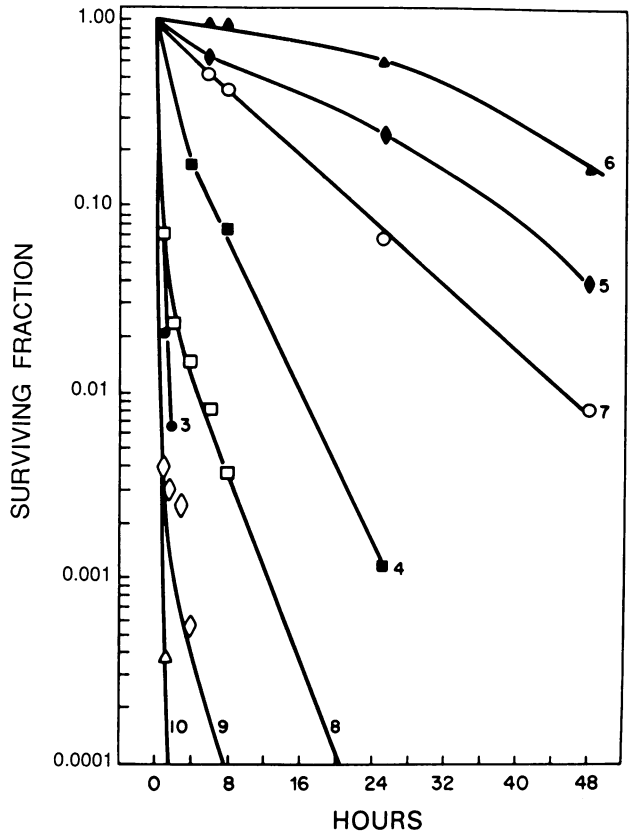

FIG. 1. Loss of MHV-A59 infectivity as a function of pH. Purified virus was incubated at 37°C in a buffer prepared from boric acid, citric acid, diethylbarbituric acid, and phosphoric acid at pHs from 3 to 10 (18) containing fetal bovine serum at a concentration of 10%. After dilution in buffer, the initial titer of virus was 5×10^5 PFU/ml. At the intervals indicated, samples were diluted into Eagle minimum essential medium containing 10% fetal bovine serum (pH 7.2) at 4°C and assayed by plaque titration (40).

min at 37°C at either pH 6.0 or pH 8.0 (Fig. 4). Whereas virions held at pH 6.0 and 4°C or 37°C, and pH 8.0 and 4°C were well dispersed over the electron microscope grid, virions treated at pH 8.0 and 37°C formed large clumps. The peplomers appeared to form lateral aggregations or tufts on the virions at pH 8.0 and 37°C, leaving large areas of viral envelope relatively free of peplomers. The sizes of the clusters of virions at pH 8.0 and 37°C were dependent on the concentration of virions (data not shown). However, since inactivation was also observed at a lower virus concentration (5×10^5 PFU/ml; Fig. 1), loss of infectivity was not solely the result of aggregation of virions but also occurred due to alteration of individual virions.

Effects of pH 8.0 and 37°C on peplomer glycoprotein. (i) Aggregation of peplomer glycoprotein on virions. In order to determine what effect pH 8.0 and 37°C might have on the viral envelope glycoproteins, virions labeled with [^{14}C]glucosamine and ^{3}H-amino acids and gradient purified at pH 6.0 and 4°C were treated at pH 8.0 for 2 h at 37 or 4°C. The virions were solubilized in SDS at 25°C and analyzed by SDS-PAGE. The [^{14}C]glucosamine profiles were plotted on the same coordinates (Fig. 5). The ^{3}H-amino acid profiles were used to confirm the alignment but are not shown in this figure. The viral glycoproteins E1 (or M) and E2 (S) can be readily identified (36). The electrophoretic mobility of the E1 glycoprotein (23K glycoprotein; fractions 63 to 73) was unchanged by treatment at a high pH. However, a significant change was detected in the E2 (S) glycoprotein. At pH 6.0 and 4 or 37°C (35) and at pH 8.0 and 4°C (Fig. 5), about 45% of the radiolabeled E2 (S) was seen as the 90K protease cleavage fragments (fractions 16 to 23), 45% of the E2 (S) was the 180K monomer (fractions 8 to 11), and about 10% was in aggregates or multimers at the top of the resolving gel (fractions 2 and 3). In contrast, on virions incubated for 2 h at pH 8.0 and 37°C, more than 80% of E2 (S) was found in large aggregates that did not enter the resolving gel (fractions 2 and 3), and only about 10% was 180K monomers and less than 5% was 90K fragments (Fig. 5). Measurement of the ratio of unaggregated (180K plus 90K) to aggregated species revealed that aggregation was maximal within 4 h of treatment at pH 8.0 and 37°C (data not shown).

(ii) Release of $E2_N$ (S1) from virions. To look more closely at the events associated with virus inactivation at pH 8.0 and 37°C, virions labeled with [^{3}H]fucose, which exclusively labels E2 (S) (36), were held at 37°C for 2 h at pH 6.0 or 8.0 and then sedimented on sucrose density gradients. Fucose-labeled virions held at pH 6.0 sedimented as a single band (Fig. 6A), but virions exposed to pH 8.0 and 37°C (Fig. 6B) released a small proportion (10%) of fucose-labeled E2 which sedimented slowly. When the released material was examined by SDS-PAGE, it was found to consist of 90K E2 (S) (Fig. 6B, inset). The 180K E2 (S) was not released from virions.

It was important to determine whether this released material consisted of a mixture of the two 90K cleavage fragments, $E2_N$ (S1) and $E2_C$ (S2), or solely of one of these subunits. To accomplish this, we treated the virions with trypsin before analysis of the effects of pH. As we showed previously, trypsin treatment of virions completely converts 180K E2 (S) to 90K species (36). Analysis of trypsin-treated virions or E2 (S) isolated from these virions more readily allowed identification of the material released and its characterization.

We previously showed that $E2_N$ (S1) can be separated from $E2_C$ (S2) by SDS-hydroxyapatite chromatography (32, 37). We used SDS-hydroxyapatite chromatography to examine the 90K material released from virions at pH 8.0 and 37°C and to compare it with defined $E2_N$ (S1) and $E2_C$ (S2) from trypsin-treated virus. From virus treated with trypsin, the 90K E2 (S) can be separated into two peaks (Fig. 7A). The first peak eluted from the column was $E2_N$ (S1) (32, 37). We demonstrated previously that this species lacked palmitic acid label (32, 37). The second peak was $E2_C$ (S2) and was previously shown to contain covalently bound palmitic acid (32, 37). Proteins isolated from these peaks have been further identified by binding of monoclonal antibodies (MAbs) (41). The SDS-hydroxyapatite chromatography profile of the 90K species released from trypsin-treated virions at pH 8.0 and 37°C was indistinguishable from that of $E2_N$ (S1) (Fig. 7B). When this released polypeptide was reacted with a panel of MAbs, it did not react with anti-$E2_C$ (S2) MAbs but did react with several anti-$E2_N$ (S1) MAbs. One anti-$E2_N$ (S1) MAb (J7.6) failed to react with the released $E2_N$ (S1) fragment (41), although it was shown to react with SDS-denatured but unreduced $E2_N$ (S1) prepared from virions. Thus the epitope recognized by J7.6 may be present but masked on the released $E2_N$ (S1) fragment.

(iii) Aggregation of isolated peplomers. In order to show that the pH-dependent reactivity of E2 (S) does not depend on other components of the virion, the effects of pH and temperature were measured on isolated peplomers.

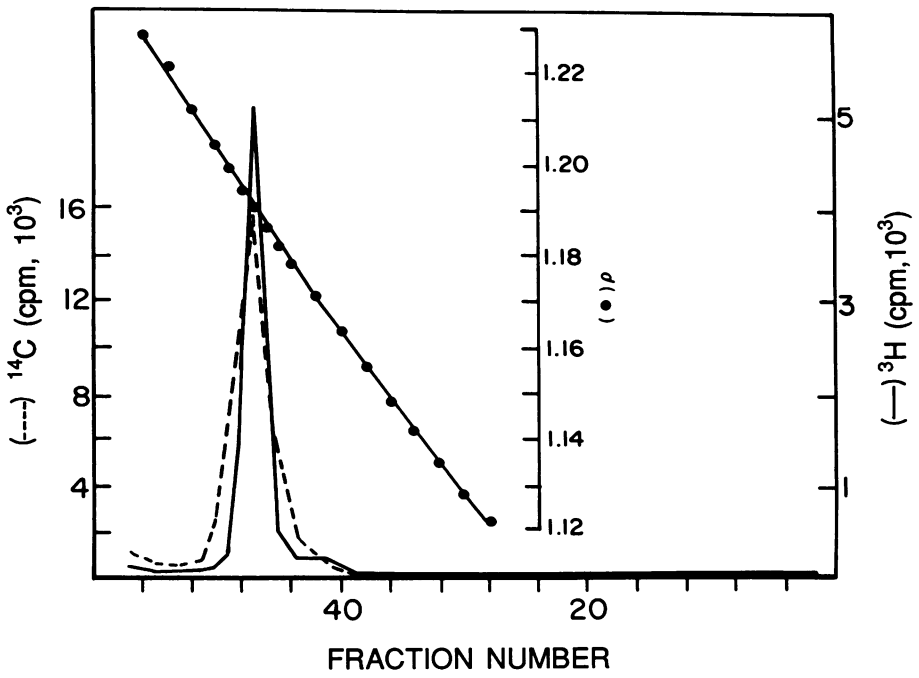

FIG. 2. Effect of pH 8.0 and 37°C treatment on the buoyant density of MHV-A59. Purified virus, labeled with [^{3}H]valine, was incubated at pH 8.0 and 37°C for 22 h, while [^{14}C]valine-labeled virus was held at pH 6.0 and 4°C. The two samples were then mixed, diluted with TMEN 6.0 buffer at 4°C, and sedimented on a 20 to 50% sucrose gradient in TMEN 6.0 at 25,000 rpm in an SW27 rotor for 21 h at 4°C.

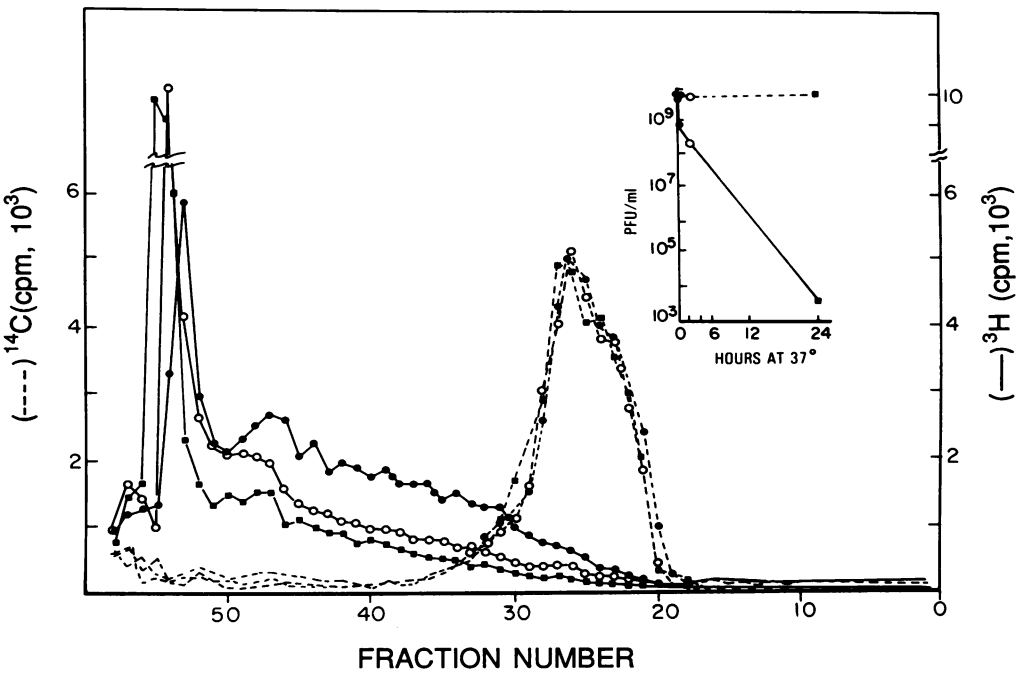

FIG. 3. Effect of pH 8.0 and 37°C treatment on MHV-A59 in velocity sedimentation. Viruses labeled with [^{3}H]valine and [^{14}C]valine were brought to pH 6.0 and 8.0, respectively, by isopycnic sedimentation into sucrose gradients in TMEN 6.0 or TMEN 8.0 buffer at 4°C. ^{3}H-labeled virus (500 μg/ml) was held at pH 6.0 and 4°C, while ^{14}C-labeled virus (500 μg/ml) was incubated at pH 8.0 and 37°C. Samples were removed at 30 min (●), 120 min (○), and 24 h (■), diluted with TMEN 6.0 at 4°C, and sedimented on 15 to 30% sucrose gradients with a cushion containing 50% sucrose in TMEN 6.0 in an SW27 rotor at 4°C for 50 min at 25,000 rpm. The distribution of virus obtained in all six gradients was plotted on a single set of coordinates. The bottoms of the gradients are at the left. (Inset) Infectivity titers of each sample before sedimentation.

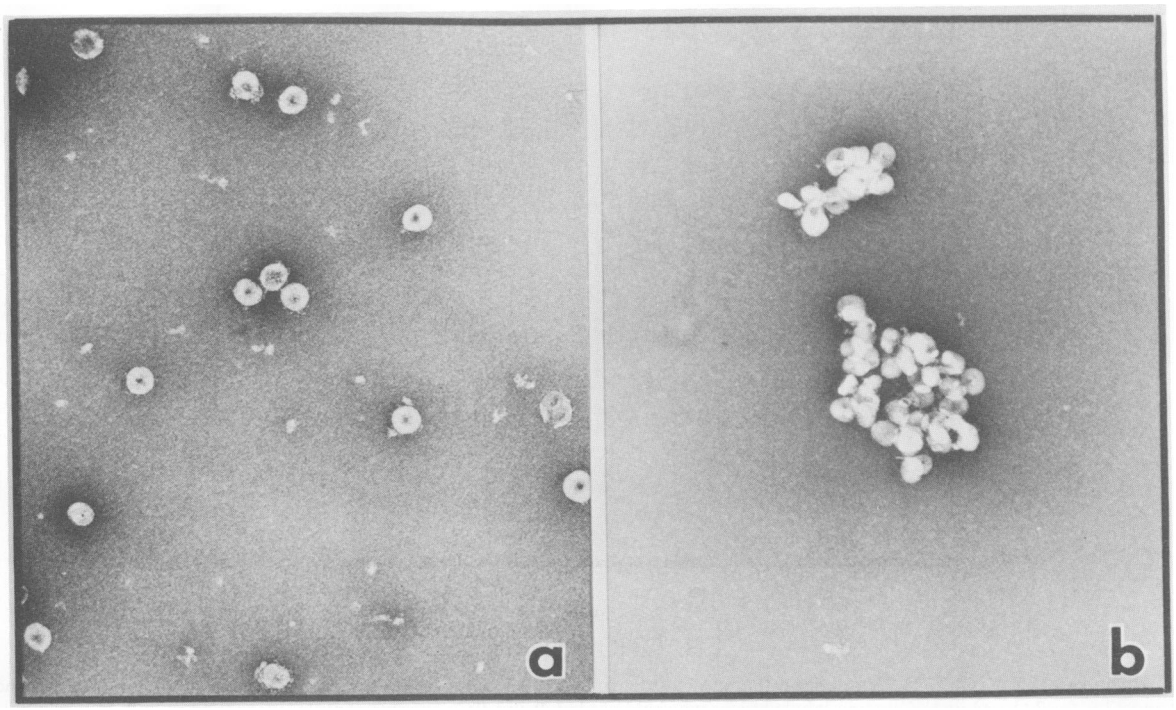

FIG. 4. Effect of pH 8.0 and 37°C treatment on the electron microscopic appearance of MHV-A59. Virions were held for 120 min at pH 6.0 and 4°C (a) or at pH 8.0 and 37°C (b).

[^{3}H]fucose-labeled spikes were solubilized from trypsin-treated virions by NP-40 and purified by sucrose density gradient ultracentrifugation (38). The purified spikes were incubated at pH 6.0 or 8.0 and 37°C and analyzed by SDS-PAGE (Fig. 8). The E2 (S) of the purified spikes migrated as a single 90K band both at the start of the experiment (Fig. 8A) and after incubation at pH 6.0 and 37°C for 20 h (Fig. 8B). However, when the 90K E2 (S) was incubated at pH 8.0 and 37°C for 4 h, 50% of the protein was held near the resolving gel (Fig. 8C). Thus, pH 8.0 induced a conformational change resulting in aggregation of approximately 50% of the 90K E2 (S). On the basis of results from other experiments (unpublished data), this protein was $E2_C$ (S2). The same result was observed with 90K E2 (S) incubated at pH 6.0 and 37°C for 20 h following dialysis against guanidine and urea in the presence of β-mercaptoethanol (Fig. 8D).

Effect of dithiothreitol. Because prolonged incubation of isolated spikes with guanidine, urea, and β-mercaptoethanol at pH 6.0 caused the same aggregation of $E2_C$ (S2) as was seen after pH 8.0 treatment at 37°C, we wondered whether the conformational changes in E2 (S) might be due to the increased reactivity of thiols at pH 8.0. To assess this possibility, purified virions were treated at pH 8.0 with 0.1 to 2.5 mM DTT and the effects on virus infectivity and E2 (S) aggregation were examined. The rate of virus inactivation at pH 8.0 and 37°C was markedly dependent on the concentration of DTT (Table 1). Aggregation of isolated spikes was also facilitated by DTT (data not shown).

pH optimum of coronavirus-induced cell fusion. Taken together, the above data indicate that profound changes are induced at pH 8.0 and 37°C in both subunits of MHV E2 (S), resulting in the aggregation of $E2_C$ (S2) and the release of $E2_N$ (S1). The biological significance of these pH-dependent changes is a question of great importance. Cell fusion

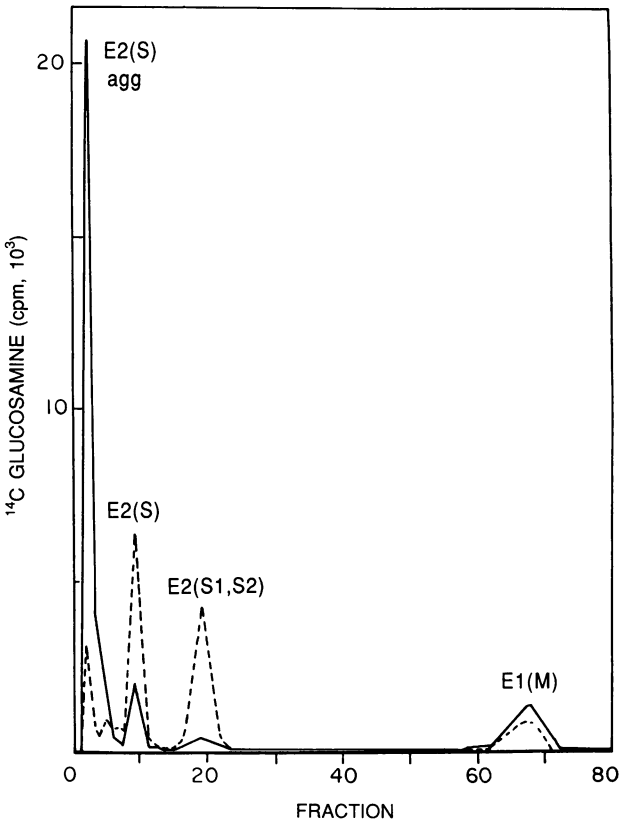

FIG. 5. SDS-PAGE of glycoproteins of pH 8.0 and 37°C-treated virus. Virus radiolabeled with glucosamine was held for 2 h at pH 8.0 and 4°C (– – –) or pH 8.0 and 37°C (——). SDS and bromophenol blue were added, and electrophoresis was performed. agg, Aggregate.

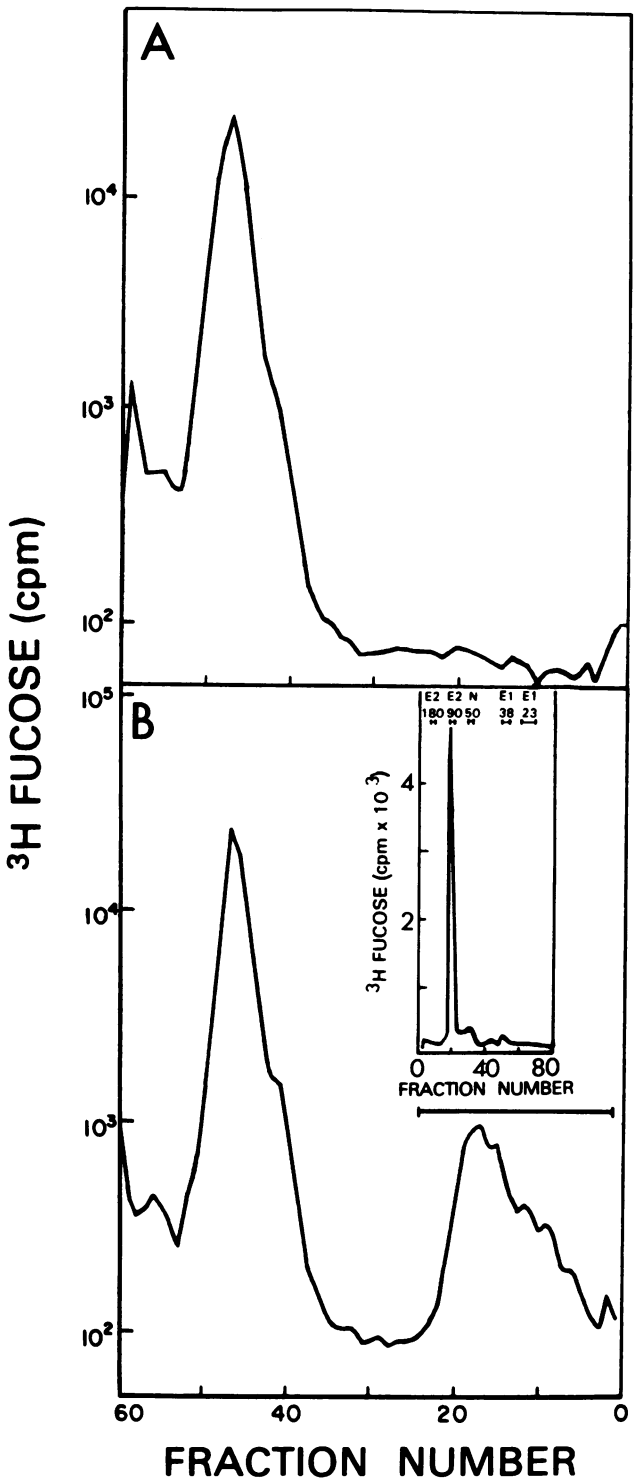

FIG. 6. Sucrose gradient of virus showing release of 90K E2 (S) at pH 8.0 and 37°C. Purified virus labeled with [^{3}H]fucose was incubated for 2 h at pH 6.0 or 8.0 and 37°C and then sedimented on 20 to 50% sucrose gradients in TMEN 6.0 buffer at 4°C. (A) pH 6.0-treated virus; (B) pH 8.0-treated virus. The bottom of the gradient is at the left. (Inset) SDS-PAGE of pooled fractions 1 to 23.

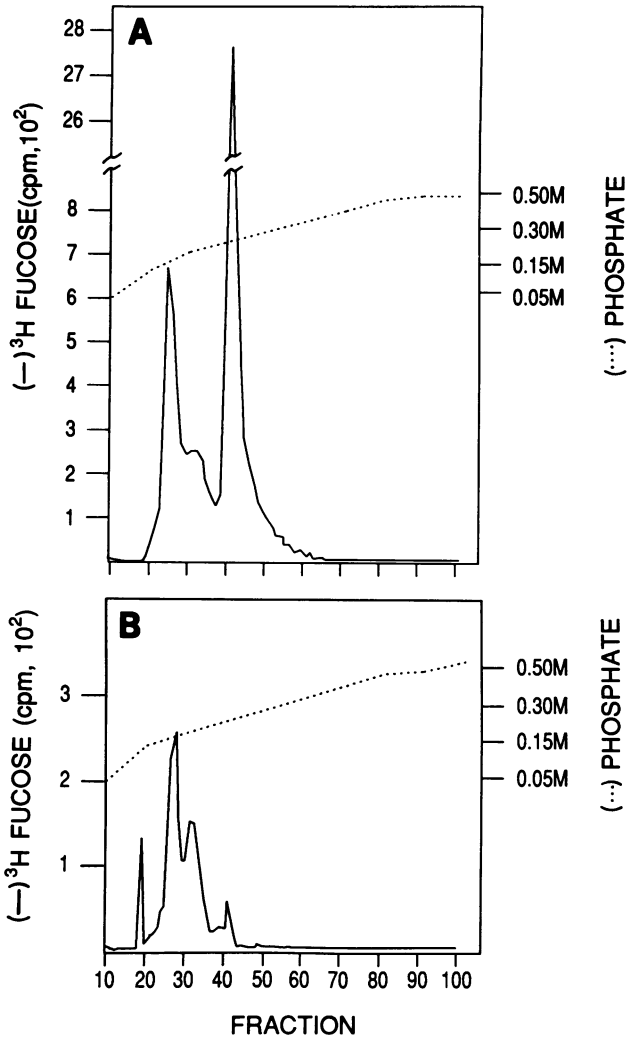

FIG. 7. Identification of pH 8.0 and 37°C-released 90K E2 (S) as $E2_N$ (S1) by high-pressure liquid chromatography on SDS-hydroxyapatite. Elution profiles of [^{3}H]fucose-labeled E2 (S) from trypsin-treated virions (A) and E2 (S) species released from virions by incubation at pH 8.0 and 37°C for 2 h (B) are shown.

induced by coronavirus MHV-A59 (fusion from within) was maximal in the range of pH 8.0 to 8.3 (Table 2). Thus, the pH at which the conformational changes occurred in E2 (S), $E2_N$ (S1), and $E2_C$ (S2) was correlated with the pH of coronavirus-induced cell fusion.

DISCUSSION

The pH-dependent thermolability of coronavirus infectivity is the result of conformational changes in the coronavirus peplomer. At pH 8.0 and 37°C the $E2_N$ (S1) subunit is released from peplomers on virions, and at least one epitope of $E2_N$ (S1) is altered during this process. At pH 8.0 and 37°C, the $E2_C$ (S2) subunit remains associated with the virus envelope and forms aggregates. These conformational changes in E2 (S) are presumed to be associated with virus entry and virus-induced fusion of target cells. Whether these biological events are mediated by aggregates of peplomers or by a small number of subunits of E2 (S) remains to be determined.

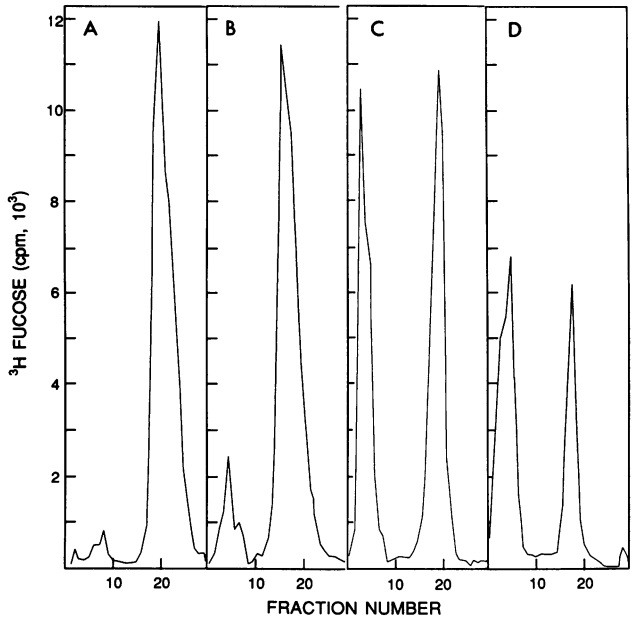

FIG. 8. Effect of pH 8.0 and 37°C treatment on aggregation of isolated E2 (S). [^{3}H]fucose-labeled E2 (S) was incubated at pH 6.0 or 8.0 and 37°C for periods of up to 20 h. Then the proteins were heated to 100°C for 1.5 min in 1% SDS with 1% β-mercaptoethanol and analyzed by SDS-PAGE. (A) Control, pH 6.0, zero time; (B) pH 6.0, 20 h; (C) pH 8.0, 4 h; (D) pH 6.0, 20 h, followed by dialysis against 7 M unbuffered guanidine and then 9 M urea with 5% β-mercaptoethanol.

The mechanisms of the conformational and functional changes in the peplomer are of great significance. On the basis of the effects of DTT on the reaction of E2 (S) at pH 8.0 and 37°C, we propose that the release of $E2_N$ (S1) and the aggregation of $E2_C$ (S2) may be triggered by rearrangement of a limited number of intramolecular disulfide bonds. The rate of virus inactivation and E2 (S) aggregation at pH 8.0 and 37°C without added reducing agent varied over a severalfold range (compare Fig. 1 and Table 1). Such variability may reflect differences in available thiols from one virus preparation to the next. The role of disulfide interchange reactions in the conformational change in E2 (S) at pH 8.0 and the relationship of intramolecular disulfide bonds to the ordered quaternary structure of the peplomer requires further examination.

A three-step process has been proposed for entry of enveloped viruses into cells (13, 28). Initially, attachment occurs between the virus receptor-binding protein and the cellular receptor. Then a conformational change exposes a hydrophobic domain in either the receptor-binding protein or another fusogenic viral envelope glycoprotein. This conformational change in the fusogenic viral protein is pH dependent, and in some cases proteolytic cleavage is necessary in order to mobilize the hydrophobic domain and activate or potentiate fusion. The third step involves an interaction between the hydrophobic domain of the fusogenic glycoprotein and a cellular membrane that results in fusion of the viral envelope and the target cell membrane, allowing entry of the viral genome into the cytoplasm. With influenza virus, for example, a conformational change in proteolytically cleaved hemagglutinin protein is triggered at pH 5.0 to 6.0. Protonation of one or more acidic residues (glutamic acid, aspartic acid, or histidine) is thought to be responsible for the conformational change that results in exposure of the fusion peptide (13). In addition, protonation of the acidic residues in the N-terminal segment of hemagglutinin 2 is required for fusion activity (27).

TABLE 1. Effect of DTT on MHV-A59 infectivity after incubation for 2 h at 37°C

DTT concn (mM)	Fraction surviving at pH:	
	8.0	6.0
0	0.37	0.96
0.1	0.095	
0.5	0.022	
2.5	0.0013	0.51

TABLE 2. Relationship between cell fusion at 8 h postinfection and pH of the medium

pH	Fusion index	
	Mock-infected cells	Infected cells
6.5	0.00	0.04
6.8	ND[a]	0.04
7.1	0.00	0.16
7.4	ND	0.29
7.7	0.00	0.35
8.0	ND	0.56
8.3	0.00	0.72

[a] ND, Not determimed.

Viruses such as influenza virus and Semliki Forest virus require an event mediated by low pH to enter the cell by fusion with the membranes of endosomes. In contrast, paramyxoviruses and lentiviruses are activated for fusion at pH 7.0 to 8.0, and these viruses may penetrate directly at the plasma membrane of the target cell. The hydrophobic sequences at the N termini of the F_1 glycoprotein of paramyxoviruses and gp41 of human immunodeficiency virus resemble the fusion peptide of influenza virus hemagglutinin (3, 12, 20).

The pH optimum of coronavirus-induced cell fusion resembles that of paramyxoviruses and lentiviruses. However, the N-terminal region of coronavirus $E2_C$ (S2) does not contain a fusion peptide analogous to those found in paramyxoviruses, lentiviruses, or myxoviruses, nor is such a sequence apparent elsewhere in $E2_C$ (S2) or $E2_N$ (S1) (24). Although proteolytic cleavage of the E2 (S) glycoprotein of MHV-A59 to yield $E2_N$ (S1) and $E2_C$ (S2) subunits has been shown to be a prerequisite for MHV fusion from without (39), the E2 (S) glycoproteins of some other coronaviruses, such as feline infectious peritonitis virus, do not require proteolytic cleavage (9). Therefore, the fusion region of coronavirus E2 is not necessarily at the amino or carboxy terminus generated by protease cleavage. It could be that either attachment of peplomers to the cellular receptor or mildly alkaline conditions can trigger the conformational changes which we have observed at pH 8.0 and 37°C, exposing a hydrophobic domain, such as that in an amphipathic helix in $E2_C$ (S2) (8; I. E. Auger and C. E. Lawrence, Comput. Appl. Biosci., in press). In contrast to this model for direct fusion of coronavirus with the plasma membrane, infectivity of MHV was also found to be reduced by pretreatment of cells with chloroquine or ammonium chloride, which altered the acidity of endosomes (21).

Functions such as receptor binding and fusion have not

yet been assigned unequivocally to either the $E2_N$ (S1) or $E2_C$ (S2) subunit. With the coronavirus infectious bronchitis virus and MHV, neutralizing antibody can be directed against either subunit (6, 22, 23, 26, 41). Cavanagh and Davis have shown that virions of infectious bronchitis virus from which S1 ($E2_N$) had been removed attached to target cells to a similar extent as did intact virions (5). Thus, S2 ($E2_C$) may contain the infectious bronchitis virus receptor-binding domain. Additional studies are required to determine which of the MHV E2 (S) subunits is involved in receptor binding and to determine the specific roles of $E2_N$ (S1) and $E2_C$ (S2) in coronavirus penetration.

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